Structural Changes in the Schiff Base Region of Squid Rhodopsin upon Photoisomerization Studied by Low-Temperature FTIR Spectroscopy†

Toru Ota,‡ Yuji Furutani,‡,§ Akihisa Terakita,§,|| Yoshinori Shichida,§,|| and Hideki Kandori*,‡,§

Department of Materials Science and Engineering, Nagoya Institute of Technology, Showa-ku, Nagoya 466-8555, Japan, Department of Biophysics, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan, and Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation, Kyoto 606-8502, Japan

Received September 24, 2005; Revised Manuscript Received December 9, 2005

ABSTRACT: Low-temperature Fourier transform infrared (FTIR) spectroscopy is used to study squid rhodopsin at 77 K in investigating structural changes in the Schiff base region upon photoisomerization. The analysis of O−D stretching vibrations in D_2O revealed that there are more internal water molecules near the retinal chromophore in squid rhodopsin than in bovine rhodopsin. Among nine O−D stretching vibrations of water in squid rhodopsin, eight peaks are identical between rhodopsin and 9-*cis*-rhodopsin (Iso). On the other hand, the isomer-specific O−D stretch of water was observed for rhodopsin (2451 cm^{-1}) and Iso (2382 cm^{-1}). Low frequencies of these bands suggest that the water forms a strong hydrogen bond with a negatively charged counterion. In addition, it was suggested that the hydrogen bond of the Schiff base is weaker in squid rhodopsin than in bacteriorhodopsin and bovine rhodopsin, and squid rhodopsin possessed similar hydrogen bonding strength for the Schiff base among rhodopsin, Iso, and bathorhodopsin. Most vibrational bands in the X−D stretch region originate from water O−D or the Schiff base N−D stretches, suggesting that the hydrogen bonding network in the Schiff base region of squid rhodopsin is composed of only water molecules. On the basis of these results, we propose that squid rhodopsin possesses a "bridge" water between the Schiff base and its counterion as well as squid retinochrome [Furutani, Y., Terakita, A., Shichida, Y., and Kandori, H. (2005) *Biochemistry 44*, 7988−7997], which is absent in vertebrate rhodopsin [Furutani, Y., Shichida, Y., and Kandori, H. (2003) *Biochemistry 42*, 9619−9625].

Vertebrate and invertebrate rhodopsins are members of the G protein-coupled receptor family that have evolved into a photoreceptive protein in visual cells (*1*−*4*). It is a membrane protein consisting of a single polypeptide opsin and a light-absorbing chromophore 11-*cis*-retinal. The opsin contains seven transmembrane α-helices, the structural motif typical of the G protein-coupled receptors, and the 11-*cis*-retinal is bound to it through the protonated Schiff base linkage (C=NH^+) with Lys296 (numbering for bovine rhodopsin) in transmembrane helix 7. Absorption of a photon by the chromophore causes isomerization to the all-*trans* form, followed by conformational changes in the protein (*5*). Metarhodopsins eventually activate the GDP−GTP exchange reaction in the trimeric G protein transducin (*6*).

It is known that the protonation state of the Schiff base in rhodopsin is stabilized by a negatively charged counterion so that the pK_a of the Schiff base is being kept higher than that in organic solvent (pK_a ∼ 7). In the case of vertebrate bovine rhodopsin, the counterion is Glu113 in helix 3 (Figure 1) (*7*−*9*). On the other hand, the corresponding amino acid residue is occupied by tyrosine in invertebrate rhodopsins, including squid and amphioxus rhodopsins. The analysis for invertebrate rhodopsins having Tyr113 by Terakita et al. (*4*) led to the conclusion that the counterion of invertebrate rhodopsins is located at Glu181 in the loop region between helices 4 and 5. The counterion of squid retinochrome having Met at position 113, a retinal photoisomerase found in cephalopod photoreceptor cells, is also Glu181 (*10*). Therefore, they concluded that the counterion of vertebrate rhodopsin is Glu113, but the rhodopsins other than vertebrate ones have a Glu181 counterion. According to the crystal structure of bovine rhodopsin, Glu181 is involved in a β-sheet constituting the retinal binding site (*11*). Through the analysis, Terakita et al. (*4*) concluded that the counterion has been evolutionally switched from position 181 to 113. In view of the pK_a of the Schiff base, there is a clear contrast between vertebrate and invertebrate rhodopsin. The pK_a in bovine rhodopsin was repoted to be >16 (*12*), being much higher than that of invertebrate rhodopsin (pK_a = 10.6) (*13*). This may be related to the different position of the counterion. It is interesting to compare the structure of the Schiff base region between vertebrate and invertebrate rhodopsins.

Internal water molecules are presumed to stabilize the ion-pair state in rhodopsins (*14*−*18*). This is indeed the case for archaeal rhodopsins such as bacteriorhodopsin (BR)[1] and *pharaonis* phoborhodopsin (*p*pR), where internal water molecules participate in the Schiff base stabilization (*19*, *20*). One water molecule bridges the Schiff base and the coun-

† This work was supported in part by grants from the Japanese Ministry of Education, Science, Sports and Culture.

* To whom correspondence should be addressed. Phone and fax: 81-52-735-5207. E-mail: kandori@nitech.ac.jp.

‡ Nagoya Institute of Technology.

§ CREST.

|| Kyoto University.

10.1021/bi051937l CCC: $33.50 © 2006 American Chemical Society
Published on Web 02/09/2006

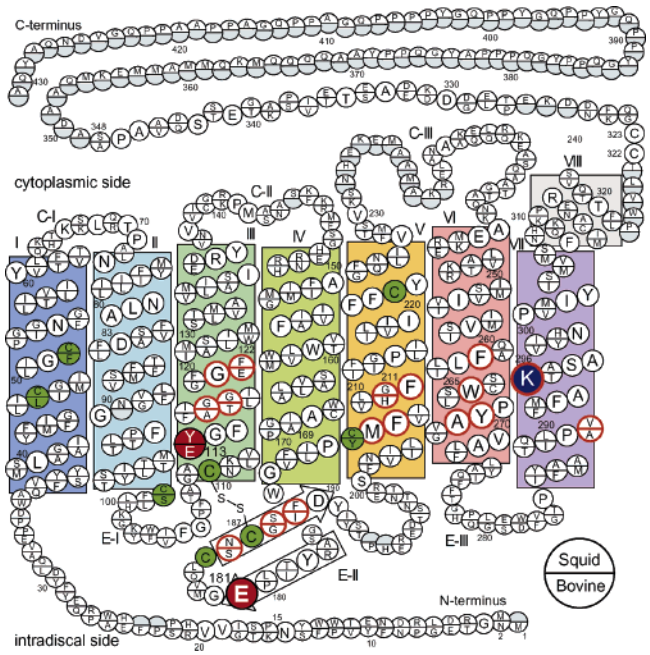

FIGURE 1: Comparison of amino acid sequences of squid and bovine rhodopsins. The transmembrane topography is based on the crystallographic three-dimensional model of bovine rhodopsin [PDB entry 1L9H (*24*)]. Single letters in circles denote residues common to squid and bovine rhodopsins. The residues that are different between squid and bovine rhodopsin are given in the top and bottom halves of the circle, respectively. The circles bisected by a red bold line compose the retinal binding site within 5 Å of the chromophore and are numbered using the bovine rhodopsin numbering system. The blue-colored circle is Lys296, which binds the retinal chromophore, while red-colored circles are Tyr113 (Glu113 for bovine) and Glu181 in squid rhodopsin, which are possible counterions. Cysteines in squid rhodopsin are represented with filled green circles.

terion in BR and *p*pR, and the low frequencies (2300−2150 cm^{-1}) of the O−D stretch of water in D_2O indicate that these water molecules form strong hydrogen bonds. The hydrogen bonding alteration of such water molecules presumably controls the proton transfer from the Schiff base to an aspartate (*21*). Previous FTIR studies of bovine rhodopsin similarly reported on the presence of internal water molecules near the retinal chromophore (*22*). However, the X-ray crystal structure of bovine rhodopsin reported the absence of such a "bridge" water between the Schiff base and Glu113 (*23*, *24*), suggesting their different static and dynamic roles between visual and archaeal rhodopsins. Similarly, our previous FTIR study of bovine rhodopsin revealed the hydrogen bonding conditions of the internal water molecule to be entirely different from those in archaeal rhodopsins (*25*). Unlike archaeal rhodopsins, water bands were not observed in the <2400 cm^{-1} region at 77 K for bovine rhodopsin (11-*cis*-retinal), Iso (9-*cis*-retinal), and Batho (all-*trans*-retinal). Together with the same results for the subsequent intermediates such as lumirhodopsin, metarhodopsin-I, and metarhodopsin-II, we concluded that the ion-pair state in bovine rhodopsin is stabilized in a manner different from that in archaeal rhodopsins (*25*).

[1] Abbreviations: BR, bacteriorhodopsin; *p*pR, *pharaonis* phoborhodopsin; Iso, isorhodopsin; Batho, bathorhodopsin; FTIR, Fourier transform infrared; PC, L-α-phosphatidylcholine; HOOP, hydrogen-out-of-plane vibration.

In this study, we extend low-temperature FTIR measurements of water molecules at 77 K to squid rhodopsin, an invertebrate rhodopsin. As a consequence, more internal water molecules were observed in squid rhodopsin than in bovine rhodopsin. Among stretching vibrations of water, we observed the isomer-specific O−D stretch in D_2O for rhodopsin (2451 cm^{-1}) and Iso (2382 cm^{-1}). The low frequencies of these bands suggest that the water forms a strong hydrogen bond with a negatively charged counterion. On the basis of the frequency of water, we propose that squid rhodopsin possesses the bridge water between the Schiff base and its counterion, which is absent in vertebrate rhodopsin (*25*).

MATERIALS AND METHODS

Squid (*Todarodes pacificus*) rhodopsin was prepared as described previously (*26*). Briefly, the purified squid rhodopsin samples were reconstituted into PC liposomes (1:100 squid rhodopsin:PC molar ratio) and dialyzed against 2 mM phosphate buffer (pH 7.5) for ~2 weeks. Then, the sample was frozen, thawed, and centrifuged at 15 000 rpm for 90 min at 4 °C. The pellet was resuspended in the same buffer, and the concentration was adjusted to ~2 absorbance units (for FTIR measurements). All experimental procedures were performed in the dark or under dim red light (>660 nm).

A 60 μL aliquot of the sample solution was deposited on a BaF_2 window with a diameter of 18 mm and dried in the glass vessel that was evacuated with an aspirator. The dry film was then hydrated by placing <1 μL of H_2O, D_2O, or $D_2{}^{18}O$ next to the film. The sample was sealed by use of another window and a rubber O-ring and mounted in an Oxford DN-1704 cryostat. The film sample was cooled ~10 min after hydration. The experimental setup was the same as that described previously (*25*), where the cryostat was mounted in a Bio-Rad FTS40 FTIR spectrometer. The cryostat was connected with an Oxford ITC-4 temperature controller, and the temperature was regulated with 0.1 K precision. The FTIR spectra were recorded with 2 cm^{-1} resolution and constructed from 128 interferograms.

The Batho-minus-rhodopsin and Batho-minus-Iso spectra were obtained by the following procedure at 77 K. Conversion of rhodopsin or Iso to Batho was achieved by illumination with 480 nm light (by use of an interference filter), while reversion of Batho to rhodopsin or Iso was achieved via illumination with >610 or >530 nm light, respectively. One hundred twenty and sixty-four independent measurements were averaged for Batho-minus-rhodopsin and Batho-minus-Iso spectra, respectively. Rhodopsin, Iso, and Batho exhibit characteristic HOOP vibrations at 971, 958, and 938 cm^{-1}, respectively, whose frequencies are very similar to those for octopus rhodopsin (*27*). We calculated the pure Batho-minus-rhodopsin and Batho-minus-Iso spectra by use of such marker bands. Linear dichroism experiments revealed the random orientation of the rhodopsin molecules in the film, so we have not applied polarized FTIR measurements.

RESULTS

Water Stretching Vibrations in the Batho-minus-Rhodopsin or -Iso Spectra. Figure 2a shows the Batho-minus-rhodopsin difference spectra in the 2730−2360 cm^{-1} region measured at 77 K. The spectrum in D_2O (red curve) exhibits peaks at 2669 (+), 2663 (−), 2657 (+), 2650 (−), 2625 (+), 2616 (−), 2607 (+), 2600 (+), 2593 (−), 2580 (+), 2571 (−),

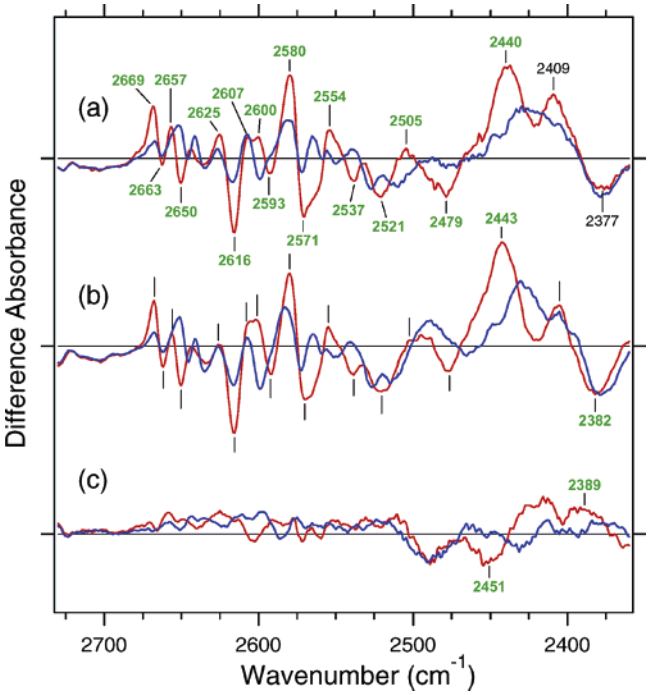

FIGURE 2: Batho-minus-rhodopsin (a), Batho-minus-Iso (b), and Iso-minus-rhodopsin (c) spectra for squid rhodopsin in the 2730−2360 cm^{-1} region. Red and blue lines represent the spectra in D_2O and $D_2{}^{18}O$, respectively. Green-labeled frequencies correspond to those identified as water stretching vibrations. One division of the *y*-axis corresponds to 0.0003 absorbance unit.

2554 (+), 2537 (−), 2521 (−), 2505 (+), 2479 (−), 2440 (+), 2409 (+), and 2377 (−) cm^{-1}, among which most of them are downshifted by 5−17 cm^{-1} for the spectrum in $D_2{}^{18}O$ (blue curve). If the normal mode of water contains only a single O−D group, the expected isotope shift is 17 cm^{-1} as for the bands at 2616 (−)/2625 (+) cm^{-1}. Coupling with other vibrations reduces the magnitude of the isotope shift so that the observed shifts exhibit large variations. The contribution of other vibrations could also affect the apparently small isotope shifts. Unlike other vibrations, the bands at 2409 (+) and 2377 (−) cm^{-1} do not exhibit a clear isotope-induced downshift. Therefore, eight negative and nine positive bands originate from O−D stretching vibrations of water. A previous study on bovine rhodopsin reported the presence of six negative and six positive bands of water at 2700−2500 cm^{-1} in the Batho-minus-rhodopsin difference spectrum (*25*). Therefore, it was suggested that there are more internal water molecules altering their hydrogen bonds upon photoisomerization in squid rhodopsin than in bovine rhodopsin.

Figure 2b shows the Batho-minus-Iso difference spectra in the same frequency region. The spectra look very similar to those of Figure 2a, indicating that the hydrogen bonding network around the retinal chromophore is similar in the 11-*cis* (rhodopsin) and 9-*cis* (Iso) forms but is altered in the all-*trans* form (Batho). This observation is consistent with the previous results for bovine rhodopsin (*25*). Only the spectral difference can be seen at <2450 cm^{-1} between panels a and b of Figure 2. Figure 2b show a positive peak at 2443 cm^{-1}, being slightly upshifted from 2440 cm^{-1} in Figure 2a. In addition, a negative peak at 2382 cm^{-1} in D_2O exhibits a clear isotope shift of water, indicating that Iso has a water O−D stretch at 2382 cm^{-1}. As a consequence, Batho-minus-Iso difference spectra possess nine negative and nine positive bands of water.

In the Batho-minus-rhodopsin and Batho-minus-Iso difference spectra, nine peaks of water were observed for Batho. While the corresponding nine peaks were observed for Iso, eight peaks were observed for rhodopsin. This may suggest that one O−D stretch of water is not clearly observed in the negative side of Figure 2a. This was indeed the case. Figure 2c shows the Iso-minus-rhodopsin difference spectra calculated from panels a and b of Figure 2. There are almost no bands at >2500 cm^{-1}, which is consistent with the same spectral feature between panels a and b of Figure 2. In contrast, some bands appeared at <2500 cm^{-1}. In particular, a negative band at 2451 cm^{-1} and a positive band at 2389 cm^{-1} exhibit the isotope shift of water, indicating that these bands originate from water O−D stretches. The 2389 cm^{-1} band probably corresponds to the band at 2382 (−) cm^{-1} in the Batho-minus-Iso spectrum (Figure 2b). In contrast, the 2451 cm^{-1} band is not clearly seen in panels a and b of Figure 2. It is likely that the Batho-minus-rhodopsin spectrum possesses a negative water band at 2451 cm^{-1}. We infer that the different positive peaks at 2440 cm^{-1} (Figure 2a) and at 2443 cm^{-1} (Figure 2b) originate from the presence of the negative peak at 2451 cm^{-1} only in Figure 2a. Thus, nineO−D stretching vibrations of water were observed for rhodopsin, Iso, and Batho.

Among nine peaks, eight peaks are identical between rhodopsin and Iso. On the other hand, rhodopsin and Iso possess a unique O−D stretching vibration at 2451 and 2382 cm^{-1}, respectively. Such an isomer-specific band was not observed for bovine rhodopsin (*25*). These frequencies are relatively low as the O−D stretch of water, indicating that the O−D group of water forms a strong hydrogen bond. The hydrogen bond is stronger in Iso than in rhodopsin. If the corresponding O−D stretch of water is at 2443 cm^{-1} for Batho, the hydrogen bonding strength of the water in Batho is comparable to that in rhodopsin. In the case of bovine rhodopsin, the lowest O−D stretch of water is at 2498 cm^{-1} (*25*).

Panels a and b of Figure 3 show the Batho-minus-rhodopsin and Batho-minus-Iso difference spectra in the 2310−1900 cm^{-1} region measured at 77 K. They clearly indicate the absence of water bands in this frequency region. We show that squid rhodopsin possesses more strongly hydrogen bonded water molecules than bovine rhodopsin. It is however noted that some archaeal rhodopsins possess water bands in this frequency region. BR exhibits three water bands at 2323, 2292, and 2171 cm^{-1} (*19*, *28*). By use of mutant proteins, we identified such O−D stretching vibrations in BR that originate from the water molecules hydrating negatively charged carboxylates (Asp85 and Asp212) (*29*, *30*). In particular, the lowest O−D stretch (2171 cm^{-1}) originates from the bridged water molecule between the protonated Schiff base and Asp85 (*29*). Thus, the hydrogen bonding strength of internal water molecules in squid rhodopsin is between those of bovine rhodopsin and BR. Recently, we reported that squid retinochrome possesses a water O−D stretch at 2334 cm^{-1} (*31*), which is close to that in squid Iso (Figure 2b). We proposed that the water molecule bridges the protonated Schiff base and Glu181, its counterion. It is likely that the water molecule in the Schiff base region possesses similar hydrogen bonding strength between squid rhodopsin and retinochrome.

Stretching Vibrations Other than Those of Water in the Batho-minus-Rhodopsin or -Iso Spectra. Panels a and b of

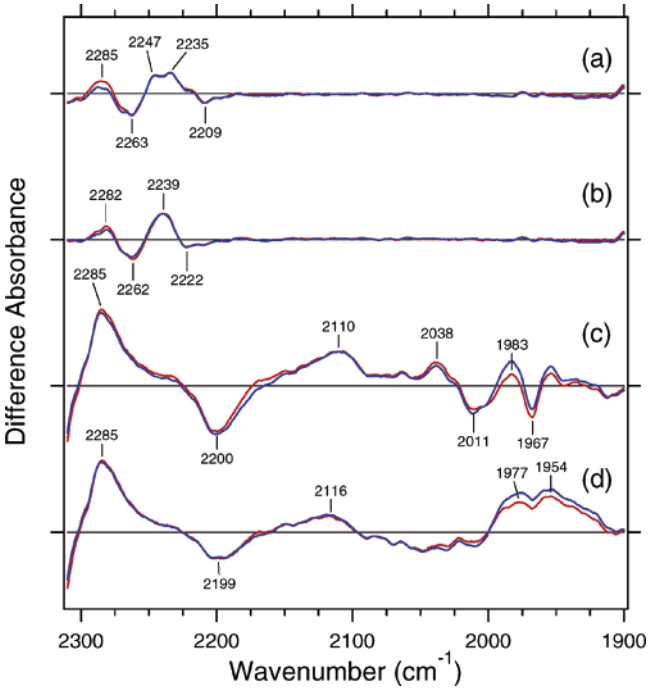

FIGURE 3: Batho-minus-rhodopsin (a) and Batho-minus-Iso (b) spectra of squid rhodopsin in the 2310−1900 cm^{-1} region are shown together with the Batho-minus-rhodopsin (c) and Batho-minus-Iso (d) spectra of bovine rhodopsin reproduced from ref *25*. Red and blue lines represent the spectra in D_2O and $D_2{}^{18}O$, respectively, where no water bands are present in this frequency region. One division of the *y*-axis corresponds to 0.0006 absorbance unit.

Figure 3 also exhibit vibrational bands other than those of water. The N−D stretching vibration of the protonated Schiff base is characteristic of this frequency region, which also provides important structural information. A previous study identified that the N−D stretching vibrations of the Schiff base in BR and *pp*R are located at 2171 and 2123 cm^{-1} (*32*) and at 2140 and 2091 cm^{-1} (*33*), respectively. Those in bovine rhodopsin are presumably located at 2011 and 1967 cm^{-1}, though not being assigned by isotope labeling (*25*). In the case of squid rhodopsin, there are no bands in the 2200−1900 cm^{-1} region (Figure 3a,b), where N−D stretches of rhodopsin and BR are present. On the other hand, the spectral feature at 2300−2200 cm^{-1} in Figure 3a is most likely to originate from the N−D stretch of the Schiff base, which is similar in shape to that at 2050−1900 cm^{-1} for bovine rhodopsin (Figure 3c) (*25*). This implies that the hydrogen bond of the Schiff base is weaker in squid rhodopsin than in BR and bovine rhodopsin. The hydrogen bonding strength of the Schiff base has been estimated by the analysis of their C═NH and C═ND stretching vibrations (*34*, *35*). While no experiments have been performed for squid rhodopsin, previous analysis of the C═N stretches of octopus rhodopsin reported that the hydrogen bond of the Schiff base is weaker in octopus rhodopsin than in bovine rhodopsin (*36*), being consistent with the conclusion presented here for squid.

In this study, we did not observe H−D exchangeable bands at 1700−1600 cm^{-1} (data not shown), implying that the C═N stretches are similar in frequency among isomeric states. This agrees with the observation for the N−D stretches. Thus, hydrogen bonding strength is not changed before and after retinal isomerization. It should be noted that the spectral feature of squid rhodopsin at 2300−2200 cm^{-1} is very similar between rhodopsin and Iso (Figure 3a,b), whereas that of bovine rhodopsin at 2050−1900 cm^{-1} is different between rhodopsin and Iso (Figure 3c,d). A previous resonance Raman study revealed that the hydrogen bonding strength of the Schiff base in bovine rhodopsin is comparable with that of Batho and stronger than that of Iso from the analysis of their C═NH and C═ND stretches (*37*). These result imply that squid rhodopsin possesses identical hydrogen bonding strength of the Schiff base between rhodopsin and Iso.

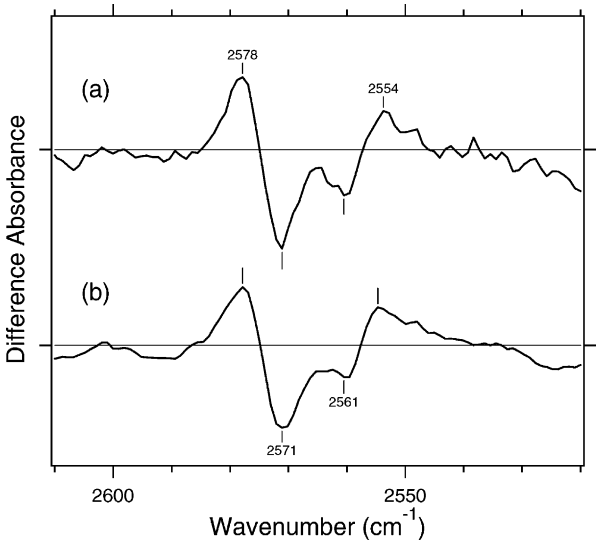

FIGURE 4: Batho-minus-rhodopsin (a) and Batho-minus-Iso (b) spectra of squid rhodopsin in the 2610−2520 cm^{-1} region, which correspond to the S−H stretching vibration of cysteine residues. The sample was hydrated with H_2O. One division of the *y*-axis corresponds to 0.00015 absorbance unit.

Another noteworthy observation for squid rhodopsin is that most spectral changes in the X−D stretching vibrations originate from either a water O−D stretch or a N−D stretch of the Schiff base. The only exceptions are the bands at 2409 (+) and 2377 (−) cm^{-1} (Figure 2a). In contrast, panels c and d of Figure 3 show the presence of bands at 2285 (+), 2200 (−), and 2110 (+) cm^{-1} for bovine rhodopsin. This fact suggests the lack of H−D exchangeable groups of protein for squid rhodopsin. Since such H−D exchangeable groups are probably located near the Schiff base region, the hydrogen bonding network in the Schiff base region of squid rhodopsin may be composed of only water molecules.

S−H Stretching Vibrations of the Cysteine Residues. Panel a and b of Figure 4 show the Batho-minus-rhodopsin and Batho-minus-Iso spectra in the 2610−2520 cm^{-1} region in H_2O, respectively. There are peaks at 2578 (+), 2571 (−), 2561 (−), and 2554 (+) cm^{-1}, which can be assigned as the S−H stretching vibrations of cysteine. The identical spectrum in D_2O indicates that the S−H group is H−D unexchangeable. In fact, no band was observed in the S−D stretching upon hydration with D_2O (data not shown). The S−H stretching frequency of cysteine appears in the 2580−2525 cm^{-1} region. Thus, the frequencies at 2571 and 2561 cm^{-1} suggest that the S−H groups of cysteines form weak hydrogen bonds.

DISCUSSION

This FTIR spectroscopic study identified the frequencies of the O−D stretching vibrations of internal water molecules

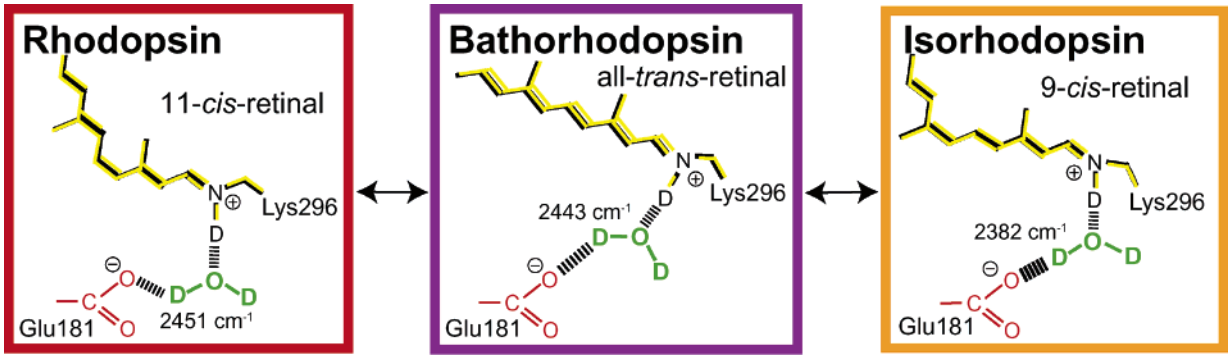

FIGURE 5: Schematic drawing of structural changes upon retinal photoisomerization in squid rhodopsin at 77 K. Glu181 is postulated to work as a counterion of the Schiff base.

in squid rhodopsin, Iso, and Batho. We observed nine O−D stretches of water with frequencies located at 2670−2370 cm^{-1}, which changed during photoisomerization processes. The numbers and frequency range are larger in squid rhodopsin than in bovine rhodopsin, suggesting stronger participation of internal water molecules. In particular, we observed an isomer-specific water molecule between rhodopsin (2451 cm^{-1}) and Iso (2382 cm^{-1}). Other water bands were identical in frequency between rhodopsin and Iso, while they change their frequencies upon formation of Batho.

The new spectra for the X−D stretching vibrations provided information about groups other than water molecules, such as the N−D stretch of the Schiff base and H−D exchangeable amide-A vibrations. Hydrogen bonding strength of the Schiff base is an important marker in the structure−function study of rhodopsins. The frequency difference of the C=N stretch in H_2O and D_2O has been regarded as the marker of the hydrogen bonding strength of the Schiff base, where a large difference corresponds to a strong hydrogen bond (*34*, *35*). The differences in octopus rhodopsin, Iso, and Batho were reported to be 36, 28, and 36 cm^{-1}, respectively (*36*). Another and more direct probe of the hydrogen bonding strength is the N−H (N−D) stretch of the Schiff base. In this study, the frequencies of the N−D stretch of the Schiff base are tentatively assigned to ∼2250 cm^{-1} for squid rhodopsin, Iso, and Batho. The frequency is greater by >250 cm^{-1} than that of bovine rhodopsin, indicating a weak hydrogen bond for squid.

No X-ray crystallographic structures have been determined for invertebrate rhodopsin, so a detailed discussion of structural changes based on the atomic structure is impossible. However, the FTIR data for the retinal, protein, and internal water molecules have been systematically obtained for squid and bovine rhodopsin, retinochrome, and archaeal rhodopsins such as BR (*19*, *20*, *25*, *31*). Therefore, on the basis of the results for water and the Schiff base, we propose a schematic model for the photoisomerization processes of squid rhodopsin (Figure 5).

The most important structural aspect is whether the bridge water is present for squid rhodopsin. The bridge water between the Schiff base and its counterion has been observed for the whole archaeal rhodopsins whose X-ray structures are known. They include BR (*38*), halorhodopsin (*39*), *pp*R (*40*, *41*), and *Anabaena* sensory rhodopsin (*42*). On the other hand, there is no bridge water for bovine rhodopsin (*11*, *24*). What is the case for squid rhodopsin? In the previous study on squid retinochrome, we proposed the presence of the bridge water between the Schiff base and its counterion, Glu181 (*31*). The experimental basis was the observation of a water molecule under strongly hydrogen bonded conditions, whose O−D stretch was at 2334 cm^{-1}. In this study of squid rhodopsin, we also observed water O−D stretches at 2451 cm^{-1} for rhodopsin and at 2382 cm^{-1} for Iso (Figure 2). This water is probably located near the chromophore of retinochrome, because its frequency change was observed at 77 K. In the model of Figure 5, we place a water molecule bridging the Schiff base and Glu181 counterion.

In contrast to the water signal, the hydrogen bond of the Schiff base seems to be similar among rhodopsin, Iso, and Batho. This fact strongly suggests that interaction between the Schiff base and the bridge water is unaltered among isomeric states. This further suggests that *cis*−*trans* isomerization of the C11=C12 or C9=C10 group does not change the geometry of the N−H (N−D) group of the Schiff base. A similar isomerization mechanism has been proposed for bovine rhodopsin (*5*, *43*). Therefore, it is likely that visual photoisomerization yields distortion of the chromophore at the center. This view is entirely different from that in archaeal rhodopsin, where photoisomerization accompanies the rotational motion of the Schiff base (*32*, *33*). As a consequence, the hydrogen bond of the Schiff base with the bridge water is broken or significantly weakened.

It is likely that the N−D stretch of the Schiff base and the O−D stretch of the bridge water are located at ∼2250 and 2451 cm^{-1}, respectively. Among isomeric states, the former frequency is unaltered, whereas the latter changes by ∼70 cm^{-1}. This may suggest that the interaction between the Schiff base and water is more predominant in stabilizing the local structure than that between the counterion and water. In the case of BR, the N−D and O−D stretching vibrations of the Schiff base and bridge water are coincident in frequency at ∼2171 cm^{-1} (*29*, *30*, *32*). The N−D and O−D stretching vibrations of the Schiff base and water at the lowest frequency are located at 2090 and 2215 cm^{-1}, respectively, for *pp*R (*33*, *44*). In contrast, the N−D and O−D stretching vibrations of the Schiff base and water at the lowest frequency are located at 2163 and 2608 cm^{-1}, respectively, for *Anabaena* sensory rhodopsin (*45*). Thus, there is no clear correlation between the N−D and O−D stretching vibrations of the Schiff base and water, respectively, for archaeal rhodopsins. Rather, the frequency of water tends to vary widely, which is consistent with the observation for squid rhodopsin presented here.

According to Terakita et al. (*4*), the counterion of the protonated Schiff base has been evolutionally switched from position 181 to 113. On the basis of the models for squid retinochrome (*31*) and rhodopsin (Figure 5), evolution of the local structure of the Schiff base region may be discussed. It is likely that both archaeal and visual rhodopsins possess a bridge water between the Schiff base and the counterion in their ancestral forms. The presence of such water molecule between the ion pair must be prerequisite for stabilization of the charged state. Then, in the process of the counterion switching from position 181 to 113, vertebrate visual pigments may acquire the mechanism for stabilizing the ion-pair state without its bridged water.

The pK_a of the Schiff base in bovine rhodopsin is >16 (*12*), while that in octopus rhodopsin is 10.5 (*13*). The difference in the Schiff base pK_a between vertebrate and invertebrate rhodopsin probably depends on the counterion position and the hydrogen bonding network around it. Although the X-ray structure of invertebrate rhodopsin has not been determined, we observed the strongly hydrogen bonded water molecule in only squid rhodopsin and retinochrome (*31*) and not in bovine rhodopsin (*25*). In the case of invertebrate rhodopsin, we proposed that a water molecule bridges the protonated Schiff base and Glu181 as shown in Figure 5. This schematic structure comes from the analogy with BR (pK_a = 13) (*46*) and *p*pR (pK_a ≥ 12) (*47*), which possess a bridged water molecule. Interestingly, hydrogen bonds of the Schiff base and the bridged water molecule are stronger in BR and *p*pR than in squid rhodopsin; the Schiff base pK_a is also higher in BR and *p*pR than in squid rhodopsin. It is thus likely that strong hydrogen bonds of not only the Schiff base but also the water molecule are prerequisite for keeping the pK_a of the Schiff base high in these systems.

On the other hand, the pK_a of the Schiff base in bovine rhodopsin is extraordinarily high (>16) (*12*), while the hydrogen bonding strength of water is weaker than in squid rhodopsin. The stabilization mechanism of the Schiff base in bovine rhodopsin seems to be different from those of BR, *p*pR, and squid rhodopsin. Recent theoretical analysis reported that the stabilization of the ion pair by water hydration was less effective than that by a hydrogen bond of Thr94 in bovine rhodopsin (*17*, *18*). The role of Thr94 in the stabilization of the protonated Schiff base was also confirmed by the site-directed mutagenesis experiment (*48*). In squid rhodopsin, Thr94 is replaced with methionine, where such a stabilization mechanism does not work. Therefore, acquirement of not only the new counterion Glu113 but also Thr94 may be important for the extremely high pK_a of the Schiff base in bovine rhodopsin. Further studies will provide a better understanding of the structure and mechanism of the pK_a control of the Schiff base in rhodopsins.

REFERENCES

1. Hubbard, R., and St. George, R. C. C. (1958) The rhodopsin system of the squid, *J. Gen. Physiol. 41*, 501−528.
2. Hofmann, K. P., and Helmreich, E. J. M. (1996) Structure and function of proteins in G-protein-coupled signal transfer, *Biochim. Biophys. Acta 1286*, 285−322.
3. Shichida, Y., and Imai, H. (1998) Visual pigment: G-protein-coupled receptor for light signals, *Cell. Mol. Life Sci. 54*, 1299−1315.
4. Terakita, A., Koyanagi, M., Tsukamoto, H., Yamashita, T., Miyata, T., and Shichida, Y. (2004) Counterion displacement in the molecular evolution of the rhodopsin family, *Nat. Struct. Mol. Biol. 11*, 284−289.
5. Kandori, H., Shichida, Y., and Yoshizawa, T. (2001) Photoisomerization in rhodopsin, *Biochemistry (Moscow) 66*, 1197−1209.
6. Hofmann, K. P. (1986) Effect of GTP on the rhodopsin-G-protein complex by transient formation of extra metarhodopsin II, *Biochim. Biophys. Acta 27*, 278−281.
7. Zhukovsky, E. A., and Oprian, K. K. (1989) Effect of carboxylic acid side chains on the absorption maximum of visual pigments, *Science 17*, 928−930.
8. Sakmar, T. P., Franke, R. R., and Khorana, H. G. (1989) Glutamic acid-113 serves as the retinylidene Schiff base counterion in bovine rhodopsin, *Proc. Natl. Acad. Sci. U.S.A. 86*, 8309−8313.
9. Nathans, J. (1990) Determinants of visual pigment absorbance: Identification for the retinylidene Schiff's base counterion in bovine rhodopsin, *Biochemistry 29*, 9746−9752.
10. Terakita, A., Yamashita, T., and Shichida, T. (2000) Highly conserved glutamic acid in the extracellular IV−V loop in rhodopsins acts as the counterion in retinochrome, a member of the rhodopsin family, *Proc. Natl. Acad. Sci. U.S.A. 97*, 14263−14267.
11. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, S. X., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor, *Science 289*, 739−745.
12. Steinberg, G., Ottolenghi, M., and Sheves, M. (1993) pK_a of the protonated Schiff base of bovine rhodopsin. A study with artificial pigments, *Biophys. J. 64*, 1499−1502.
13. Liang, J., Steinberg, G., Livnah, N., Sheves, M., Ebrey, T. G., and Tsuda, M. (1994) The pK_a of the protonated Schiff bases of gecko cone and octopus visual pigments, *Biophys. J. 67*, 848−854.
14. Gat, Y., and Sheves, M. (1993) A mechanism for controlling the pK_a of the retinal protonated Schiff base in retinal proteins. A study with model compounds, *J. Am. Chem. Soc. 115*, 3772−3773.
15. Han, M., and Smith, S. O. (1995) NMR constraints on the location of the retinal chromophore in rhodopsin and bathorhodopsin, *Biochemistry 34*, 1425−1432.
16. Hudson, B. C., and Birge, R. R. (1999) Angular orientation of the retinyl chromophore of bacteriorhodopsin: Reconciliation of ^{2}H NMR and optical measurements, *J. Phys. Chem. A 103*, 2274−2281.
17. Buss, V., Sugihara, M., Entel, P., and Hafner, J. (2003) Thr94 and Wat2b effect protonation of the retinal chromophore in rhodopsin, *Angew. Chem., Int. Ed. 42*, 3245−3247.
18. Sugihara, M., Buss, V., Entel, P., and Hafner, J. (2004) The nature of the complex counterion of the chromophore in rhodopsin, *J. Phys. Chem. B 108*, 3673−3680.
19. Kandori, H. (2000) Role of internal water molecules in bacteriorhodopsin, *Biochim. Biophys. Acta 1460*, 177−191.
20. Furutani, Y., and Kandori, H. (2002) Internal water molecules of archaeal rhodopsins, *Mol. Membr. Biol. 19*, 257−265.
21. Tanimoto, T., Furutani, Y., and Kandori, H. (2003) Structural changes of water in the Schiff base region of bacteriorhodopsin: Proposal of a hydration switch model, *Biochemistry 42*, 2300−2306.
22. Nagata, T., Terakita, A., Kandori, H., Kojima, D., Shichida, Y., and Maeda, A. (1998) The hydrogen-bonding network of water molecules and the peptide backbone in the region connecting Asp83, Gly120, and Glu113 in bovine rhodopsin, *Biochemistry 37*, 17216−17222.
23. Teller, D. C., Okada, T., Behnke, C. A., Palczewski, K., and Stenkamp, R. E. (2001) Advances in determination of a high-resolution three-dimensional structure of rhodopsin, a model of G-protein-coupled receptors (GPCRs), *Biochemistry 40*, 7761−7772.
24. Okada, T., Fujiyoshi, Y., Silow, M., Navarro, J., Landau, E. M., and Shichida, Y. (2002) Functional role of internal water molecules in rhodopsin revealed by X-ray crystallography, *Proc. Natl. Acad. Sci. U.S.A. 99*, 5982−5987.
25. Furutani, Y., Shichida, Y., and Kandori, H. (2003) Structural changes of water molecules during the photoactivation processes in bovine rhodopsin, *Biochemistry 42*, 9619−9625.
26. Maeda, A., Shichida, Y., and Yoshizawa, T. (1979) Formation of 7-cis- and 13-cis-retinal pigments by irradiating squid rhodopsin,

Biochemistry 18, 1449−1453.
27. Nishimura, S., Kandori, H., Nakagawa, M., Tsuda, M., and Maeda, A. (1997) Structural dynamics of water and the peptide backbone around the Schiff base associated with the light-activated process of octopus rhodopsin, *Biochemistry 36*, 864−870.
28. Kandori, H., and Shichida, Y. (2000) Direct observation of the bridged water stretching vibrations inside a protein, *J. Am. Chem. Soc. 122*, 11745−11746.
29. Shibata, M., Tanimoto, T., and Kandori, H. (2003) Water molecules in the Schiff base region of bacteriorhodopsin, *J. Am. Chem. Soc. 125*, 13312−13313.
30. Shibata, M., and Kandori, H. (2005) FTIR studies of internal water molecules in the Schiff base region of bacteriorhodopsin, *Biochemistry 44*, 7406−7413.
31. Furutani, Y., Terakita, A., Shichida, Y., and Kandori, H. (2005) FTIR studies of the photoactivation processes in squid retinochrome, *Biochemistry 44*, 7988−7997.
32. Kandori, H., Belenky, M., and Herzfeld, J. (2002) Vibrational frequency and dipolar orientation of the protonated Schiff base in bacteriorhodopsin before and after photoisomerization, *Biochemistry 41*, 6026−6031.
33. Shimono, K., Furutani, Y., Kamo, N., and Kandori, H. (2003) Vibrational modes of the protonated Schiff base in *pharaonis* phoborhodopsin, *Biochemistry 42*, 7801−7806.
34. Rodman-Gilson, H. S., Honig, B., Croteau, A., Zarrilli, G., and Nakanishi, K. (1998) Analysis of the factors that influence the C═N stretching frequency of polyene Schiff bases. Implications for bacteriorhodopsin and rhodopsin, *Biophys. J. 53*, 261−269.
35. Baasov, T., Friedman, N., and Sheves, M. (1987) Factors affecting the C═N stretching in protonated retinal Schiff base: A model study for bacteriorhodopsin and visual pigments, *Biochemistry 26*, 3210−3217.
36. Huang, L., Deng, H., Weng, G., Koutalos, Y., Ebrey, T., Groesbeek, M., Lugtenburg, J., Tsuda, M., and Callender, R. H. (1996) A resonance Raman study of the C═N configurations of octopus rhodopsin, bathorhodopsin, and isorhodopsin, *Biochemistry 35*, 8504−8510.
37. Palings, I., Pardoen, J. A., van den Berg, E., Winkel, C., Lugtenburg, J., and Mathies, R. A. (1987) Assignment of fingerprint vibrations in the resonance Raman spectra of rhodopsin, isorhodopsin, and bathorhodopsin: Implications for chromophore structure and environment, *Biochemistry 26*, 2544−2556.
38. Luecke, H., Schobert, B., Richter, H. T., Cartailler, J. P., and Lanyi, J. K. (1999) Structure of bacteriorhodopsin at 1.55 Å resolution, *J. Mol. Biol. 291*, 899−911.
39. Kolbe, M., Besir, H., Essen, L. O., and Oesterhelt, D. (2000) Structure of the light-driven chloride pump halorhodopsin at 1.8 Å resolution, *Science 288*, 1390−1396.
40. Luecke, H., Schobert, B., Lanyi, J. K., Spudich, E. N., and Spudich, J. L. (2001) Crystal structure of sensory rhodopsin II at 2.4 angstroms: Insights into color tuning and transducer interaction, *Science 293*, 1499−1503.
41. Royant, A., Nollert, P., Edman, K., Neutze, R., Landau, E. M., Pebay-Peyroula, E., and Navarro, J. (2001) X-ray structure of sensory rhodopsin II at 2.1-Å resolution, *Proc. Natl. Acad. Sci. U.S.A. 98*, 10131−10136.
42. Vogeley, L., Sineshchekov, O. A., Trivedi, V. D., Sasaki, J., Spudich, J. L., and Luecke, H. (2004) *Anabaena* sensory rhodopsin: A photochromic color sensor at 2.0 Å, *Science 306*, 1390−1393.
43. Mathies, R. A., and Lugtenburg, J. (2000) The primary photoreaction of rhodopsin, in *Handbook of Biological Physics* (Stavenga, D. G., de Grip, W. J., and Pugh, E. N., Eds.) Vol. 3, pp 55−90, Elsevier, Amsterdam.
44. Kandori, H., Furutani, Y., Shimono, K., Shichida, Y., and Kamo, N. (2001) Internal water molecules of *pharaonis* phoborhodopsin studied by low-temperature infrared spectroscopy, *Biochemistry 40*, 15693−15698.
45. Furutani, Y., Kawanabe, A., Jung, K.-H., and Kandori, H. (2005) FTIR spectroscopy of the all-trans form of *Anabaena* sensory rhodopsin at 77 K: Hydrogen bond of a water between the Schiff base and Asp75, *Biochemistry 44*, 12287−12296.
46. Druckmann, S., Ottolenghi, M., Pande, A., Pande, J., and Callender, R. H. (1982) Acid−base equilibrium of the Schiff base in bacteriorhodopsin, *Biochemistry 21*, 4953−4959.
47. Iwamoto, M., Furutani, Y., Sudo, Y., Shimono, K., Kandori, H., and Kamo, N. (2002) Role of Asp193 in chromophore-protein interaction of *pharaonis* phoborhodopsin (sensory rhodopsin II), *Biophys. J. 83*, 1130−1135.
48. Janz, J. M., and Farrens, D. L. (2004) Role of the retinal hydrogen bond network in rhodopsin Schiff base stability and hydrolysis, *J. Biol. Chem. 279*, 55886−55894.

BI051937L